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Citation: Jones, Amanda, Sutcliffe, Iain and Goodfellow, Michael (2012) *Prescottia equi* gen. nov., comb. nov.: a new home for an old pathogen. *Antonie van Leeuwenhoek*, 103 (3). pp. 655-671. ISSN 0003-6072

Published by: Springer

URL: <http://dx.doi.org/10.1007/s10482-012-9850-8> <<http://dx.doi.org/10.1007/s10482-012-9850-8>>

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1 *Prescottia equi* gen. nov., comb. nov.: a new home for an old pathogen

2

3 Amanda L. Jones^{1,2}, Iain C. Sutcliffe¹ and Michael Goodfellow²

4

5 ¹School of Life Sciences, Northumbria University, Newcastle upon Tyne, NE1 8ST,
6 UK

7 ²School of Biology, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK

8

9

10 Author for correspondence: Amanda L. Jones. Telephone: +44(0)191-2274895; Fax:
11 +44(0)191-2273519; E-mail: Amanda.L.Jones@northumbria.ac.uk

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14 Subject category: New Taxa; Actinobacteria.

15

16

17 Running Title: *Prescottia equi* gen. nov., comb. nov.

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22

23 **Abstract:**

24 The taxonomic status of *Rhodococcus equi*, originally isolated from foal specimens,
25 has been the subject of discussion for a number of years. The chequered history of the
26 taxon has prompted this polyphasic analysis of *R. equi* strains, close members of the
27 genus *Rhodococcus* and representatives of other genera classified in the order
28 *Corynebacteriales*, to establish the taxonomic position of this taxon. Thirty one *R.*
29 *equi* strains, including the type strain, were examined for genotypic and numerical
30 taxonomic properties. The resultant data are consistent with their classification in the
31 order *Corynebacteriales*, but the *R. equi* strains formed a distinct phyletic clade away
32 from representatives of other members of the genus *Rhodococcus* in the 16S rRNA
33 gene tree. Representatives of this clade shared their highest pairwise 16S rRNA gene
34 sequence similarities with the type strain of *Rhodococcus kunmingensis* (95.2 to
35 98.1%). However, the *R. equi* taxon was readily distinguished from *R. kunmingensis*
36 and from the other members of the order *Corynebacteriales* using a combination of
37 genotypic, chemotypic and phenotypic properties. On the basis of these data the *R.*
38 *equi* strains are considered to represent a new genus. The name proposed for this
39 taxon is *Prescottia* gen. nov., with *Prescottia equi* comb. nov. as the type species
40 containing the type strain, C 7^T (=ATCC 25729^T=ATCC 6939^T=CCUG
41 892^T=CIP 54.72^T=DSM 20307^T=HAMBI 2061^T=NBRC
42 14956^T=JCM 1311^T=JCM 3209^T=LMG 18452^T=NBRC 101255^T
43 =NCTC 1621^T=NRRL B-16538^T=VKM Ac-953^T).

45 **Introduction**

46
47 In 1923, Magnusson isolated a strain of the primary causal agent of equine pneumonia
48 and classified it in the genus *Corynebacterium* as *Corynebacterium equi* (Magnusson
49 1923). Subsequently, following a somewhat turbulent taxonomic history (Barton and
50 Hughes 1980; Goodfellow and Jones 2012), a study by Goodfellow & Alderson
51 (Goodfellow and Alderson 1977) led to the species being transferred to the genus
52 *Rhodococcus* as *Rhodococcus equi*. The organism, a facultative intracellular parasite
53 of macrophages, is an important pathogen of foals; it causes fatal lymphadenitis and
54 ulcerative enteritis in 3 to 5 month old foals (Meijer and Prescott 2004; Giguere et al.
55 2011a; Giguere et al. 2011b; Prescott 1991).

57 More recently, *R. equi* has been recognised as an opportunistic pathogen of humans,
58 especially immunocompromised patients; necrotizing pneumonia is the most common
59 disease manifestation although the organism also causes extra-pulmonary infections
60 (Kedlaya et al. 2001; Yamshchikov et al. 2010; Takai et al. 1994; Prescott 1991).
61 Misidentification of *R. equi* strains in animals and humans as mycobacterial infections
62 (Meijer and Prescott 2004) delays correct treatment of patients thereby causing
63 relapses and fatalities, with mortality rates between 50-55% in patients with HIV, 20-
64 25% in those with non-HIV compromised immunity and 11% in immunocompetent
65 patients (Kedlaya et al. 2001). The importance of *R. equi* as a multihost pathogen has
66 led to extensive studies of its virulence (von Bargen and Haas 2009; Giguere et al.
67 2011a) and to sequencing of the whole genome of a pathogenic strain (Letek et al.
68 2010).

69

70 The genus *Rhodococcus* currently encompasses 33 species with validly published
71 names. Previous studies have shown that members of the genus can be assigned to
72 three 16S rRNA gene clades which fall within the evolutionary radiation of the order
73 *Corynebacteriales* (Jones and Goodfellow. 2012; Goodfellow and Jones 2012),
74 namely the *R. equi*, *Rhodococcus erythropolis* and *Rhodococcus rhodochrous* clades
75 (Goodfellow et al. 1998; McMinn et al. 2000; Jones et al. 2004; Jones and
76 Goodfellow. 2012); the taxonomic integrity of these taxa are supported by specific
77 16S rRNA gene sequences (Goodfellow et al. 1998; Gurtler et al. 2004). The aim of
78 the present polyphasic taxonomic study was to establish whether strains falling within
79 the *R. equi* clade merit generic status.

80

81 **Materials and methods**

82

83 Thirty one *R. equi* strains, 15 strains representing other *Rhodococcus* species, along
84 with representatives from the related mycolic acid containing genera
85 *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Tsukamurella* and
86 *Williamsia*, including 59 type strains, were obtained from either private or public
87 culture collections (Table S1). The organisms were maintained on glucose-yeast
88 extract agar (GYEA; Gordon and Mihm 1962) at room temperature and as glycerol
89 suspensions (20%, v/v) at -20°C. Fifteen strains were randomly selected as duplicates
90 to establish test error in the numerical taxonomic and molecular fingerprinting studies.

91 Extraction of chromosomal DNA from each of the 129 isolates was carried out using
92 the method of Kim et al. (1998). The strains were the subject of a composite DNA
93 fingerprinting analysis based on two different repetitive DNA elements (rep-PCR;
94 Versalovic et al. 1994) using BOX A1R (Pathom-aree et al. 2006) and ERIC
95 (Versalovic et al. 1991) primer sets, and three individual amplified 16S ribosomal
96 DNA restriction studies (ARDRA; Vaneechoutte et al. 1995) that used the restriction
97 enzymes *Alu* I, *Hpa* II and *Cfr* I31.

98

99 The *rep*-PCR amplifications were performed in a 25 µl reaction mixture containing 1
100 µl template DNA (100ng), 1 x PCR buffer, 4 µM of primer BOXA1R (Pathom-aree et
101 al. 2006) with 2 µM of primers ERIC 1R and ERIC 2 (Versalovic et al. 1991), 100
102 mM DMSO, 6 mM MgCl₂, 0.1 U BioTaq DNA polymerase and 1.24 mM of each of
103 the four dNTPs. Amplification with the primers was carried under the following
104 conditions: initial denaturation step at 95°C for 5 minutes, 30 cycles of 95°C for 1
105 minute, 52°C for 1 minute and 65°C for 8 minutes with a final incubation at 65°C for
106 18 minutes. PCR amplifications of 16S rRNA genes were carried out according to the
107 method of Kim et al. (Kim et al. 1998), with each PCR product digested singly for 2
108 hours at 37°C in final reaction volumes of 10 µl, consisting of 8.5 µl of PCR product,
109 1 µl of 1 x buffer Y⁺/Tango™ and 0.5 U of restriction enzyme.

110

111 Cluster analysis of the *rep*-PCR fingerprints was based on band intensity using the
112 unweighted-pair-group method with averages algorithm (UPGMA; Sokal and
113 Michener 1958) and the Pearson's product-movement correlation coefficient (Pearson
114 1926). Each primer type was analysed separately. The similarity of the ARDRA band
115 patterns generated by the digest with each restriction endonuclease were analysed
116 separately using the UPGMA algorithm (Sokal and Michener 1958) and the Jaccard
117 coefficient (Jaccard 1908). The *rep*-PCR and ARDRA fingerprint data were combined
118 to give a consensus matrix.

119

120 The 129 strains and the 15 control duplicated cultures were examined for 96 unit
121 characters (Table 2) using methods known to yield data of value for the classification
122 and identification of mycolic acid-containing actinomycetes (Goodfellow and
123 Alderson 1977; Goodfellow et al. 1998; Goodfellow et al. 1982a; Goodfellow et al.
124 1982b; Jones et al. 2008; Goodfellow and Jones 2012). Tolerance to antibiotics was

125 determined using antibiotic discs (Table 2; Oxoid Ltd., Wade Road, Basingstoke, UK)
126 impregnated with specific concentrations of antibiotic in accordance with the British
127 Society of Antimicrobial Chemotherapy (BSAC) guidelines (Andrews 2001). When
128 zone sizes were equal to or greater than those specified by the BSAC guidelines, a
129 negative (sensitive) result was scored; zone sizes measuring less than those specified
130 by the guidelines were scored positive (resistant).

131

132 The test strains were also examined for their capacity to cleave a range of fluorogenic
133 and chromogenic substrates (Table 2). The substrates were dissolved in double
134 strength phosphate buffer to give a final concentration of 1 mM. Suspensions of two
135 day old biomass cultured on GYEA were suspended to give a McFarland density of 3.
136 Equal volumes of each suspension and substrate solution were dispensed into
137 microtitre wells. The plates containing the fluorogenic substrates were read for
138 fluorescence at excitation 365 nm, emission 440 nm and sensitivity 28; the
139 chromogenic substrates were examined for absorbance at the wavelength of 405 nm.
140 The resultant readings were recorded as time zero and after two days incubation at
141 30°C, the microtitre plates were read again at the same settings. Results of the tests
142 were tabulated in the Microsoft Excel program (Microsoft Co., Seattle, USA) and
143 transformed into two mutually exclusive states, scored positive (1) or negative (0), for
144 the numerical taxonomic analysis. The tests were coded positive when the difference
145 in fluorescent/absorbance intensities between the test and negative controls was more
146 than 0 [$R_p = V_r - V_c - V_{a+b}$ (R_p , positive result; V_r , resultant reaction between test
147 strain and conjugated substrate; V_c , value of cell inoculum alone; V_{a+b} , value of
148 organism free control)].

149

150 **Results**

151

152

153

154 The results of the remaining phenotypic tests in the data matrix were converted to
 155 binary format (1/0) written to a NTS file using Programmer's File Editor (PFE)
 156 software. The final dataset was the subject of cluster analyses using the NTSYSp
 157 program (version 2.0; Numerical Taxonomy and Multivariate Analysis System; Rohlf
 158 1998). Similarity values were calculated using the S_{SM} coefficient and clustering
 159 accomplished with the UPGMA algorithm (Sneath and Sokal 1973); the results were
 160 presented as a dendrogram. Co-phenetic correlation values (Sokal and Rohlf 1962)
 161 were calculated using the NTSYS 'Coph' and 'Mxcomp' functions to estimate how
 162 well the structure inherent in the similarity matrix was preserved by the clustering
 163 procedure.

164

165 The phylogenetic positions of 26 *R. equi* isolates were determined in a 16S rRNA
 166 gene sequence analysis. PCR amplification and direct sequencing of the purified
 167 products were carried out using the method of Kim et al. (Kim et al. 1998) The almost
 168 complete 16S rRNA gene sequences were aligned with corresponding sequences of
 169 representatives of genera classified in the order *Corynebacteriales* (retrieved from the
 170 DDBJ/EMBL/GenBank databases), using the CLUSTAL W alignment option and 16S
 171 rRNA secondary structural information held in the MEGA 5 program (Tamura et al.
 172 2011). Phylogenetic trees were inferred using the neighbor-joining (Saitou and Nei
 173 1987), least squares (Fitch and Margoliash 1967), maximum-parsimony (Kluge and
 174 Farris 1969) and maximum-likelihood (Felsenstein 1981) tree-making algorithms
 175 from the MEGA 5 program (Tamura et al. 2011) and evolutionary distance matrices
 176 prepared after Jukes and Cantor (1969). The resultant unrooted tree topologies were
 177 evaluated in a bootstrap analysis (Felsenstein 1985) based on 1,000 resamplings.
 178 Accession numbers for the sequences generated in this study are listed in Fig 1.

179

180 Heterogeneity within *Rhodococcus* was confirmed by comparison of 16S rRNA gene
 181 sequences. Almost complete 16S rRNA gene sequences (1202 to 1518 nucleotides
 182 [nt]) were generated for 26 of the *R. equi* strains. The phylogenetic relationships
 183 between these organisms, the type strains of the other *Rhodococcus* species and
 184 representatives of the 15 other genera classified in the order *Corynebacteriales* are
 185 shown in Fig 1. The *R. equi* strains formed a distinct clade that was supported by all
 186 four tree-making algorithms and by a bootstrap value of 88%, a result that is in good
 187 agreement with corresponding data from previous studies (Goodfellow et al. 1998;

188 McMinn et al. 2000) and is underpinned by the study of Rainey et al. (Rainey et al.
189 1995), which also demonstrated the separation of *R. equi* from other members of the
190 genus *Rhodococcus*. Nearly all of the remaining rhodococci were assigned either to
191 the *R. erythropolis* or *R. rhodochrous* clades, a result in line with those from earlier
192 studies (McMinn et al. 2000; Jones and Goodfellow. 2012; Goodfellow et al. 1998;
193 Gurtler et al. 2004). The separation of the *R. equi* strains from these and related taxa is
194 supported by specific nucleotide 16S rRNA gene signatures (Table 3). All of these
195 results are consistent with the view that *R. equi* merits generic status (Jones and
196 Goodfellow. 2012).

197

198 The 29 *R. equi* strains formed a distinct taxon based on the molecular fingerprint and
199 numerical taxonomic data. The consensus dendrogram derived from the analyses of
200 the *rep*-PCR and ARDRA fingerprint data (Fig 2) showed that the *R. equi* strains
201 formed a distinct taxon that lay between corresponding groups encompassing
202 members of the *R. erythropolis* and *R. rhodochrous* phyletic lines. All of these taxa
203 were sharply separated from each other and from the representatives of mycolic acid-
204 containing bacteria classified in the genera *Corynebacterium*, *Dietzia*,
205 *Mycobacterium*, *Nocardia*, *Tsukamurella* and *Williamsia*. Similarly, the *R. equi* strains
206 formed a distinct cluster defined at the 84.5 % similarity level in the complementary
207 numerical taxonomic study (Fig 3). Once again, this taxon was distinct from members
208 of the *R. erythropolis* and *R. rhodochrous* groups and from clusters composed of
209 representatives of the other mycolic acid-containing genera. It is evident from both
210 analyses that the genus *Rhodococcus* is a polyphyletic taxon, as highlighted in an
211 earlier study (Rainey et al. 1995). Confidence can be placed in the present results as
212 the duplicated *Rhodococcus* strains clustered together in the molecular fingerprint
213 analysis while the test error recorded for these strains in the numerical taxonomic
214 study was low (3.6%), a result well below the 10% cut off recommended previously
215 (Sneath and Johnson 1972). The cophenetic correlation value obtained in the S_{SM}-
216 UPGMA analysis was high at 0.72.

217

218 **Discussion**

219

220 Cumulatively, the chemotaxonomic, molecular systematic and numerical taxonomic
221 data show that *R. equi* can be distinguished readily from other members of the genus

222 *Rhodococcus* and from the remaining genera classified in the order *Corynebacteriales*
223 (Table 4). Consequently, we propose that *R. equi* be reclassified as a new genus,
224 named *Prescottia* with *Prescottia equi* as the type species.

225

226 This change will still enable medical and veterinary clinical diagnosis to proceed with
227 caution using the same diagnostic criteria as currently used for *R. equi* (Giguere and
228 Prescott 1997; Prescott 1991). As *R. equi* is the only documented *Rhodococcus*
229 species to contain the virulence plasmids which encode the VapA protein (in foal and
230 some human isolates) or the homologous VapB protein (in porcine and human
231 isolates) (Giguere et al. 2011a; Tkachuk-Saad and Prescott 1991; Giguere et al.
232 2011b), PCR amplification of the *vapA* gene for equine isolates (Giguere et al. 2011b)
233 and/or of the *vapB* gene for isolates from human clinical sources are thus of major
234 diagnostic importance. Moreover, the numerical taxonomic analysis revealed
235 phenotypic characteristics of diagnostic potential for the recognition of *R. equi* strains
236 (Table 5). All 31 *R. equi* strains, including the type strain, were positive in the forty
237 two enzyme tests listed in Table 5 with the exception of strain N1310 which was
238 unable to hydrolyse L-arginine-7AMC, L-glycine-7AMC-hydrogen bromide or L-
239 ornithine-7AMC-dihydrochloride. Integration of the tests summarised in Table 5 into
240 diagnostic keys should facilitate the improved recognition of this pathogen.

241

242 The degree of confidence that can be placed in a classification is reflected in the
243 congruence found when the same strains are assigned to corresponding taxa based on
244 different, but complementary, taxonomic criteria. It is, therefore, encouraging that in
245 the present study that the *R. equi* strains were assigned to taxa distinct from other
246 rhodococci based on both the molecular fingerprint and numerical taxonomic data
247 (Figs 2 and 3). Excellent congruence was found between the composition of the two
248 *R. equi* subgroups recovered in these analyses though an exception was *R. equi*
249 N1310, which was recovered in subgroup 1 in the former analysis and in subcluster 2
250 in the latter one. However, in each analysis the type strain of *R. equi* and 15 related
251 strains, including isolates from animal, human and environmental sources (Table S1),
252 formed a homogeneous taxon, the taxonomic status of which is underpinned by
253 pyrolysis mass spectrometric and previous numerical phenetic data (Goodfellow and
254 Alderson 1977; Goodfellow et al. 1982a; Goodfellow et al. 1982b; McMinn et al.
255 2000).

256

257 It can be concluded from the genotypic and phenotypic data that the taxon containing
 258 isolate C 7^T corresponds to *R. equi* (Goodfellow et al. 1998; Magnusson 1923). It is,
 259 therefore, proposed that this species be recognised as the type species of the new
 260 genus *Prescottia*. However, additional work needs to be carried out to establish
 261 whether the subgroup 2 strains should be recognised as a second *Prescottia* species.
 262 Further work is also required to clarify the taxonomic status of *R. kunmingensis* DSM
 263 45001^T which lies towards the periphery of the *Prescottia* 16S rRNA gene clade (Fig.
 264 1). The type strains of *R. equi* and *R. kunmingensis* share a DNA:DNA relatedness
 265 value of $34.4 \pm 10\%$ (Wang et al. 2008), a value well below the cut-off point
 266 recommended for the delineation of bacterial species (Wayne et al. 1987).

267

268 **Description of *Prescottia* gen. nov.**

269 *Prescottia* (Pres.cot'ti.a. fem. n. *Prescottia*) named after John Prescott to celebrate his
 270 many contributions towards unravelling the pathogenicity of *Rhodococcus equi*.

271

272 The description is based on data taken from this and previous studies (Collins et al.
 273 1982a; Collins et al. 1982b; Collins et al. 1977; Collins et al. 1979; Collins et al. 1985;
 274 Cummins and Harris 1956; Goodfellow and Alderson 1977; Keddie and Cure 1977;
 275 Komura et al. 1975; Nishiuchi et al. 2000; Schleifer and Kandler 1972; Uchida and
 276 Aida 1977; Mordarski et al. 1980b; Mordarski et al. 1980a; Zakrzewska-Czerwinska
 277 et al. 1988) (Table 4).

278

279 Aerobic, Gram-positive, acid-alcohol fast, nonmotile, pleomorphic actinomycete
 280 which may show traces of elementary branching at early stages of growth. Whole-
 281 organism hydrolysates are rich in 2, 6-diaminopimelic acid, arabinose and galactose.
 282 The peptidoglycan is of the A1 γ type. Muramic acid moieties are N-glycolated. Cells
 283 contain diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and
 284 phosphatidylinositol mannosides as major polar lipids; complex mixtures of straight
 285 chain saturated, monounsaturated and branched chain fatty acids and dihydrogenated
 286 menaquinones with eight isoprene units as the predominant isoprenologue. Mycolic
 287 acids have 28 to 50 carbon atoms and up to four double bonds. The fatty acids
 288 released on pyrolysis gas chromatography of mycolic acid esters have 12 to 16 carbon
 289 atoms. The DNA G + C content ranges from 69-72 mol%. The genus *Prescottia*, as
 290 determined by 16S rDNA gene sequence analyses, is a member of the order
 291 *Corynebacteriales*.

292

293 Isolated from soil and intestinal tracts and faeces of animal species. Causes equine
 294 pneumonia in foals and is an opportunistic pathogen of humans.

295

296 **Description of *Prescottia equi* comb nov.**

297 *Prescottia equi* (e'qui. L. n. *equus* horse; L. gen. n. of the horse).

298

299 In addition to the characteristics given in the genus description, the species has the
 300 following properties based on the results of this and previous studies (Barton and
 301 Hughes 1980; Goodfellow and Alderson 1977; Goodfellow et al. 1982a; Mordarski et
 302 al. 1980b; Mordarski et al. 1980a).

303

304 In smears cells often show a clumping or palisade arrangement or L- or V- shaped
 305 elements. Smears from liquid cultures sometimes show branching filamentous forms
 306 with swollen ends. Smooth, shiny, pale pink colonies with entire margins are
 307 produced on glucose-yeast extract agar; abundant slime which may drop onto the lids
 308 of inverted Petri dishes may be produced during incubation. Grows from 5 to 40°C,
 309 optimally around 30°C. Phosphatase positive but negative for arbutin and esculin
 310 hydrolysis. Reduction of nitrate to nitrite and hydrolysis of urea are variable. Degrades
 311 Tweens 20, 40, 60 and 80 but not arbutin, cellulose, chitin, guanine, hypoxanthine, L-
 312 tyrosine, uric acid or xanthine. Degradation of adenine is variable. Cleaves the
 313 following exopeptides: D-alanine-7-AMC trifluoroacetate, L-arginine-7-AMC, L-
 314 glutamate 7-AMC, L-glycine 7-AMC- hydrogen bromide, L-histidiine 7-AMC, L-*iso*-
 315 leucine 7-AMC-trifluoroacetate, L-leucine-7-AMC, L-lysine 7-AMC-acetate, L-
 316 methionine 7-AMC-acetate, L-ornithine-7-AMC dihydrochloride, L-phenylalanine 7-
 317 AMC-trifluoroacetate, L-proline-7-AMC hydrogen bromide, L-threonine 7-AMC, L-
 318 tyrosine 7-AMC and N-benzyloxycarbonyl glycyl-prolyl-7-AMC. Cleaves the
 319 following glycosides: 4MU-acetyl-β-D-glucosaminide, 4MU-β-D-fucoside, 4MU-β-
 320 D-galactoside, 4MU-α-D-glucoside, 4MU-β-D-glucoside, 4MU-β-D-glucuronide,
 321 4MU-α-D-mannopyranoside, 4MU-β-D-ribofuranoside and 4MU-B-D-
 322 xylanopyranoside. Cleaves the following inorganic esters: dihydroumbelliferone, 4-
 323 methyl-7-nitrocoumarin, 4MU-phosphate disodium salt, organic esters, 4MU-acetate,
 324 4MU-butyrate, 4MU-elaidate, 4MU-*p*-guanidinobenzoate, 4MU-heptanoate, 4MU-
 325 laurate, 4MU-nonanoate, 4MU-propionate and 4MU-stearate. In addition, the

326 chromogenic substrates *o*-nitrophenyl-myrisate, *p*-nitrophenyl- α -L-fucoside, *p*-
327 nitrophenyl-phenylphosphonate, *p*-nitrophenyl-phosphorylcholine, *p*-nitrophenyl- α -L-
328 rhannopyranoside and *p*-nitrophenol- α -L-xylopyranoside are cleaved.

329

330 Grows on adonitol, amygdalin, L-arabinose, cellobiose, erythritol, glycerol, glycogen,
331 inositol, inulin, maltose, mannitol, α - and β -methyl-D-glucoside, raffinose, rhamnose,
332 salicin, sorbitol, sucrose, trehalose and xylose as sole carbon sources but not on
333 xylitol. Sensitive to (μ g ml): cephalexin (30), clindamycin (2), colistin sulphate (25),
334 cotrioxide (25), fusidic acid (10), nalidixic acid (30), novobiocin (5) and penicillin G,
335 but is resistant to erythromycin (4), gentamicin (8), lincomycin (64), minocycline
336 (0.125), neomycin (8), novobiocin (4), streptomycin (4) and tobramycin (8).

337

338 The DNA G + C content of the type strain is 70.4 mol%.

339

340 The type strain, C 7^T (= ATCC 25729^T = ATCC 6939^T = CCUG 892^T = CIP 54.72^T =
341 DSM 20307^T = HAMBI 2061^T = NBRC 14956^T = JCM 1311^T = JCM 3209^T = LMG
342 18452^T = NBRC 101255^T = NCTC 1621^T = NRRL B-16538^T = VKM Ac-953^T) was
343 isolated from a lung abscess of a foal.

344

345 **Acknowledgements**

346

347 Amanda Jones is grateful to the Freeman Hospital, Newcastle upon Tyne and to the
348 School of Biology, Newcastle University, Newcastle upon Tyne for financial support.
349 The authors are indebted to Dr Jean Euzéby for helping to name the new taxon, Prof.
350 Arthur James for his expertise in fluorogenic and chromogenic substrates and Prof.
351 John D. Perry for helping with the antibiotic tolerance studies.

352

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354

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 557

Unit character	Unit character
A. Biochemical tests (% w/v):	D. Enzyme tests
Aesculin (0.1)*, ²	Cleavage of 7-amino-4-methylcoumarin substrates (7AMC):
Arbutin (0.1)*, ²	Exopeptidases:
Nitrate reduction (0.1)	D-alanine-7AMC-trifluoroacetate ³
Nitrite reduction (0.1)	L-arginine-7AMC ³
Urease production (2.0) ²	L-asparagine-7AMC-trifluoroacetate ³
B. Degradation tests (% w/v):	L-aspartate-7AMC ³
Adenine (0.4)*, ²	L-glutamate-7AMC ³
Casein (0.1)*, ²	L-glycine-7AMC-hydrogen bromide ³
DNA (0.3)*, ²	L-histidine-7AMC ³
Guanine (0.4)*, ²	<i>iso</i> -L-leucine-7AMC-trifluoroacetate ³
Hypoxanthine (0.4)*, ²	L-leucine-7AMC ³
RNA (0.3)*, ²	L-lysine-7AMC-acetate ³
Starch (0.1)*, ²	L-methionine-7AMC-acetate ³
Tributyrin (1.0 %)*, ²	L-ornithine-7AMC-dihydrochloride ³
L-Tyrosine (0.5)*, ²	L-phenylalanine-7AMC-trifluoroacetate ³
Tween 20 (1.0 %)*, ²	L-proline-7AMC-hydrogen bromide ³
Tween 40 (1.0 %)*, ²	L-pyroglutamate-7AMC ³
Tween 60 (1.0 %)*, ²	L-threonine-7AMC ³
Tween 80 (1.0 %)*, ²	L-tyrosine-7AMC ³
Uric acid (0.5)*, ²	N-benzoyloxycarbonyl-arginine-7AMC-hydrochloride ³
Xanthine (0.4)*, ²	N-benzoyloxycarbonyl-glycyl-prolyl-7AMC ³
C. Morphological tests	Cleavage of 4-methylumbelliferone substrates (4MU):
Growth on glucose-yeast extract agar:	Glycosides:
Light orange*	4MU-N-acetyl-β-D-glucosaminide ³
Light yellow pink*	4MU-α-L-arabinopyranoside ³
Pale orange yellow*	4MU-β-D-cellobioside ³
Slight yellow pink*	4MU-β-D-fucoside ³
Very orange*	4MU-α-D-galactopyranoside ³
Very red*	4MU-β-D-galactoside ³
Very yellow*	4MU-α-D-glucoside ³
Yellow white*	
Mucoid*	
Rough*	
Smooth*	

Unit character	Unit character
4MU-β-D-glucoside ³	<i>p</i> -np phenyl phosphonoate ³
4MU-β-D-glucuronide ³	<i>p</i> -nitrophenyl phosphorylcholine ³
4MU-α-D-mannopyranoside ³	
4MU-β-D-mannopyranoside ³	E. Tolerance tests
4MU-β-D-ribofuranoside ³	Sensitivity to antibiotics (µg/ml):
4MU-β-D-xylopyranoside ³	Aminocoumarin:
Inorganic esters:	Novobiocin (5) ¹
8-acetyl-7-hydroxy-4-methylcoumarin ³	Cephalosporin:
Dihydroumbelliferone ³	Cephalexin (30) ¹
4-methyl-7-nitrocoumarin ³	Fusidane:
4MU-phosphate disodium salt ³	Fusidic acid (10) ¹
4MU-sulphate potassium salt ³	Glycopeptides and peptide:
Organic esters:	Bacitracin (10) ¹
4MU-acetate ³	Macrolide:
4MU-butyrate ³	Erythromycin (5) ¹
4MU-elaidate ³	Penicillin:
4MU- <i>p</i> -guanidinobenzoate ³	Penicillin G (1) ¹
4MU-heptanoate ³	Polymyxin:
4MU-laurate ³	Colistin sulphate (25) ¹
4MU-nonanoate (genzyme) ³	Quinolone:
4MU-palmitate ³	Ciprofloxacin (1) ¹
4MU-propionate ³	Nalidixic acid (30) ¹
4MU-stearate ³	Tetracycline:
Cleavage of nitrophenol (np):	Tetracycline hydrochloride (10) ¹
Glycosides:	Miscellaneous:
<i>p</i> -np-α-L-fucoside ³	Clindamycin (2) ¹
<i>p</i> -np-α-L-rhamnopyranoside ³	Cotrimaxazole (25) ¹
<i>p</i> -np-α-L-xylopyranoside ³	
Inorganic esters:	
<i>o</i> -np myrisate ³	

* Tests carried out using a multipoint inoculator (Goodfellow et al. 1992); Tests carried out using 0.5¹; 2.5² and 3.0³ McFarland inoculum densities.

Table 3. Nucleotide signatures that separate *R. equi* from closely related *Rhodococcus* clades defined in the 16S rRNA sequence analysis^a

Position ^b	<i>Nocardia</i> spp.	<i>R. corynebacterioides</i>	<i>R. equi</i>	<i>R. erythropolis</i>	<i>R. kunmingensis</i>	<i>R. rhodochrous</i>
40-42	G:S	G:Y	G:S	G:C	G:S	G:C
43	K	G	G	G	G	G
44	Y	Y	U	U	U	U
64-65	W:R	A:A	A:A	A:G	A:G	A:R
70	H	W	U	U	U	U
75	S	-	C	C	C	C
78	Y	-	-	-	-	A
84-88	T:C	C:C	Y:G	K:Y	C:C	Y:B
102	C	U	Y	C	C	Y
122	C	A:C	A:C	A:C	C:N	A:C
127-139	Y:Y	G:C	K:C	G:C	G:C	G:C
182-185	-	C:U	G:C	-	G:U	-
191-196	-	C:U	G:G	-	U:G	-
199	K	G	U	G	U	G
207	D	A	G	R	A	R
216	R	A	A	A	A	W
217-218	Y:Y	U:C	C:U	Y:Y	Y:Y	Y:Y
223-224	Y:R	C:A	C:A	C:A	C:A	C:A
292	R	G	G	A	G	G
303	Y	C	Y	U	C	Y
380-384	R:C	C:C	A:C	R:C	C:C	M:C
459	H	Y	A	N	C	M
490	U	C	C	C	U	B
579	U	C	C	C	C	C
610-615	-	C:A	U:G	-	U:G	C:A
623-627	-	-	C:A	-	-	-
634	R	A	G	A	G	A
760	A	G	G	G	G	G
835-835	-	-	U:C	-	-	-
998	C	C	U	Y	C	Y
100-1008	C:Y	C:C	C:C	Y:Y	C:C	C:Y
1017-1023	R:Y	C:C	C:C	B:Y	C:C	R:Y
1040	-	G	A	R	G	R
1119-1121	Y:R	C:G	C:G	U:A	U:A	Y:R
1132-1139	R:Y	R:Y	G:C	R:Y	G:C	R:B
1150-1152	Y:R	C:G	C:G	U:A	U:A	Y:R
1335	Y	C	C	C	C	C

^aDegenerate nucleotide codes: R (A+G), Y (C+T), M (A+C), S (G+C), W (A+T), B (not A), N (any), K (G+T).

^bNumbering based on *Escherichia coli*.

Table 4. Characteristics of wall chemotype IV genera classified in the order *Corynebacteriales*

Characters	Genera																
	<i>Prescottia</i>	<i>Amycolicoccus</i>	<i>Corynebacterium</i>	<i>Dietzia</i>	<i>Gordonia</i>	<i>Hoyosella</i>	<i>Millisia</i>	<i>Mycobacterium</i>	<i>Nocardia</i>	<i>Rhodococcus</i>	<i>Segniliparus</i>	<i>Skermania</i>	<i>Smaragdicooccus</i>	<i>Tomitella</i>	<i>Tsakmurella</i>	<i>Turicella</i>	<i>Williamsia</i>
Cell morphology	Rod-coccus growth cycle, with trace elements of branching	Coccoid cells	Pleomorphic rods, often club-shaped; commonly in angular and palisade arrangement	Short rods and cocci	Rods and cocci or moderately branching hyphae	Coccoid cells occurring singly, in pairs, in tetrads or in small clumps	Characteristic rudimentary right angled branching	Rods, occasionally branched filaments which fragment into rods and coccoid elements	Mycelium which fragments into rods and cocci	Rods to extensive substrate mycelium; the latter fragments into irregular rods and cocci	Rods	Mycelium resembling a pine tree	Coccoid cells	Irregular rods that exhibit snapping division. coccoid rods apparent after prolonged culture	Rods occur singly, in pairs or in masses; coccobacillary forms occur	Single cell, arranged in V-forms or palisades	Thin irregular rods or cocci occur singly or in small clusters
Aerial hyphae	Absent	Absent	Absent	Absent	Absent	ND	Present	Usually absent	Present	Absent	Absent	Present but not visible to the naked eye 9-21	Absent	Absent	Absent	Absent	Present
Growth of visible colonies (days)	1-2	1-2	1-2	1-3	1-3	1-3	1-3	2-40	1-5	1-3	3-4		7	ND	1-3	1-2	1-4
Acid-fastness	Acid-alcohol-fast	ND	Sometimes weakly acid-fast	Not acid fast	Partially acid-alcohol-fast	weakly acid-fast	Acid-alcohol-fast	Strongly acid-fast	Partially acid-fast	Partially acid-fast at some stage of the growth cycle	Acid-alcohol-fast	Not acid fast	ND	ND	Partially acid-alcohol-fast	ND	ND
Strictly aerobic	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Fatty acid composition ^a	S, U, T, T16 ^g	S, U	S, U ^b	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U, T	S, U	S, U	S, U, T	S, U, T	S, U, T
Major menaquinone(s) ^c	MK-8(H ₂)	MK-7, MK-8	MK-8(H ₂)	MK-8(H ₂)	MK-9(H ₂)	MK-8	MK-8(H ₂)	MK-9(H ₂)	MK-8(H ₄ , ω-cycl) ^f	MK-8(H ₂)	ND	MK-8(H ₄ , ω-cycl)	SQA-8(H ₄ ω cycl) and SQB (H ₄ dicycl)	MK-9(H ₂)	MK-9	MK-10, MK-11	MK-9(H ₂)
Muramic acid type	Glycolated	ND	Acetylated	Acetylated	Glycolated	Acetylated	Glycolated	Glycolated	Glycolated	Glycolated	ND	Glycolated	Glycolated	Glycolated	Glycolated	ND	Glycolated
Mycolic acid pattern ^d	Single spot	None	Single spot	Single spot	Single spot	ND	Single spot	Multiple spots	Single spot	Single spot	Multiple spots	Single spot	ND	ND	Two spots	None	Single spot
Mycolic acids:																	
Overall size (number of carbons)	28-50	ND	22-38	34-38	46-77	ND	44-52	60-90	46-64	30-54	ND	58-64	43-49	42-52	62-78	ND	50-56
Number of double bonds	0-4	ND	0-2	0-1	1-6	ND	ND	1-4	0-3	0-4	ND	2-6	ND	ND	1-7	ND	ND
Fatty acids released on pyrolysis	12-16	ND	8-18	ND	16-18	ND	ND	22-26	12-18	12-16	ND	16-20	ND	ND	22-26	ND	ND
Phosphatidylethanolamine present in polar lipid patterns	+	+	- ^f	+	+	+	+	+	+	+	ND	+	+	+	+	ND	+
DNA G + C content (mol%)	69-72 ^h	60.04	51-67	66-73	63-69	49.3	64.7	57-73	63-72	63-73	68-72	67.5	63.7	69.3-71.6	68-78	65-72	64-65

Symbols: +, positive; -, negative; and ND, not determined. Data taken from Adachi *et al.* (Adachi et al. 2007), Butler *et al.* (Butler et al. 2005), Cummins *et al.* (Cummins and Harris 1956), Keddie *et al.* (Keddie and Cure 1977), Komura *et al.* (Komura et al. 1975), Schleifer *et al.* (Schleifer and Kandler 1972), Soddell *et al.* (Soddell et al. 2006) and Uchida *et al.* (Uchida and Aida 1977). ^a, Abbreviations: S, straight chain; U, unsaturated; T, tuberculostearic acid (10-methyloctadecanoic acid). ^b, *C. bovis*, *C. minutissimum*, *C. urealyticum* and *C. variabile* contain tuberculostearic acid (Collins et al. 1982a; Kämpfer et al. 1999; Lechevalier et al. 1977). ^c, Examples of abbreviations: MK-9(H₂), menaquinone with two of the nine isoprene units hydrogenated (Collins et al. 1979; Collins et al. 1985; Collins et al. 1977); SQA and SQB, smaradiquinones A and B. ^d, Number of mycolic acid spots produced from whole-organism methanolystaes (Minnikin et al. 1980; Minnikin et al. 1993; Minnikin et al. 1975; Barton et al. 1989; Collins et al. 1982a; Hsu et al. 2011; Nishiuchi et al. 2000). ^e, In mycobacterial mycolic acids, double bonds may be converted to cyclopropane rings; methyl branches and other oxygen functions may be present. ^f, Present in *Corynebacterium bovis* and *C. urealyticum* (Kämpfer *et al.*, (Kämpfer et al. 1999)). ^g, carbon 16 version of 10-methyloctadecanoic acid present in half of the *Prescottia* strains. ^h DNA G+C content of *P. equi* (Mordarski et al. 1980b; Mordarski et al. 1980a; Zakrzewska-Czerwinska et al. 1988)

Table 5. Key Phenotypic Characteristics of *R. equi* strains from subclusters 1 and 2.

Tests	Subcluster 1 and 2 N = 31
Biochemical:	
Arbutin	~*
Nitrite	-
Degradation:	
Casein	-
Guanine	-
Hypoxanthine	-
Tween 20	+
Tween 60	+
Tween 80	+
Xanthine	-
Enzyme tests:	
L-Arginine-7AMC	~*
L-Glutamate-7AMC	+
L-Glycine-7AMC-hydrogen bromide	~*
L-Histidine-7AMC	+
Iso-L-Leucine-7AMC-trifluoroacetate	+
L-Leucine-7AMC	+
L-Lysine-7AMC-acetate	+
L-Methionine-7AMC-acetate	+
L-Ornithine-7AMC-dihydrochloride	~*
L-Phenylalanine-7AMC-trifluoroacetate	+
L-Proline-7AMC-hydrogen bromide	+
L-Threonine-7AMC	+
L-Tyrosine-7AMC	+
Z-Glycyl-Prolyl-7AMC	+
4MU-N-acetyl-β-D-glucosaminide	+
4MU-β-D-fucoside	+
4MU-β-D-galactoside	+
4MU-α-D-glucoside	+
4MU-β-D-glucoside	+
4MU-β-D-glucuronide	+
4MU-α-D-mannopyranoside	+
4MU-β-D-ribofuranoside	+
4MU-β-D-xylopyranoside	+
Dihydroumbelliferone	+
4-methyl-7-nitrocoumarin	+
4mu-phosphate disodium salt	+
4mu-acetate	+
4mu-butyrate	+
4mu-elaidate	+
4mu-p-guanidinobenzoate	+
4mu-heptanoate	+
4mu-laurate	+
4mu-nonanoate (genzyme)	+
4mu-propionate	+
4mu-stearate	+
p-np-α-L-fucoside	+
p-np-α-L-rhamnopyranoside	+
p-np-α-L-xylopyranoside	+
o-np myrisate	+
p-np phenyl phosphonate	+
p-nitrophenyl phosphorylcholine	+
Tolerance tests:	
Penicillin	+
Clindamycin	+

Symbols: +, positive; -, negative; *, a single strain from subcluster 2 (N1310) produced the opposite result to that recorded.

Legend for Figures

Fig. 1. Neighbor-joining tree (Saitou and Nei 1987) based on a nearly complete 16S rRNA gene sequences showing the position of the *R. equi* strains and representatives of genera classified in the order *Corynebacteriales*. Asterisks indicate branches of the tree that were also found with the least-squares (Fitch and Margoliash 1967) maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) tree-making algorithms; the symbols F, L and P indicate branches recovered using the least-squares, maximum-likelihood and maximum-parsimony methods, respectively. The numbers at the nodes indicate levels of bootstrap support based on a neighbor-joining analysis of 1000 re-sampled datasets; only values above 50% are given. The scale bar indicates 10 substitutions per nucleotide position. ^T, type strain.

Fig. 2. A consensus dendrogram of the representatives of genera classified in the order *Corynebacteriales* based on the Pearson-UPGMA (Pearson 1926) and S_J-UPGMA (Jaccard 1908; Sokal and Michener 1958) analysis of the *rep*-PCR and ARDRA fingerprints showing relationships between the *R. equi* isolates and reference strains SMC, single-membered cluster.

Fig. 3. Dendrogram showing relationships between the *R. equi* isolates and the representatives of other mycolic acid containing taxa defined in the S_{SM}-UPGMA analysis.





